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Transgenic mice expressing either the neu or TGFα in the mammary epithelium develop focal mammary tumours that occur only after a long latency period. Because the epidermal growth factor receptor and Neu are capable of forming heterodimers that are responsive to EFGR ligands such as TGFα, we examined whether Neu and TGFα could cooperate to accelerate the onset of mammary tumours. To accomplish this objective we have interbred separate strains harbouring either a MMTV/TGFα or MMTV/neu transgenes to generate bitransgenic mice coexpressing both Neu and TGFα. The results revealed that by contrast to either parental strain, female mice coexpressing TGFα and Neu in the mammary epithelium develop multifocal mammary tumours which arise after a short latency period. The rapid induction of mammary tumours in these bitransgenic mice is further correlated with the tyrosine phosphorylation of both Neu and EGFR. Moreover, in established cell lines overexpressing EGFR, EGF stimulation results in the transphosphorylation of the Neu and the formation of complexes between c-Src and tyrosine phosphorylated Neu. Taken together these observations suggest that Neu and EGFR cooperate by recruiting distinct but complementary signalling pathways.

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INTRODUCTION

The Epidermal Growth Factor Receptor (EGFR) family comprises four closely related type 1 receptor tyrosine kinases (RTKs) termed EGFR, Neu (c-erbB-2,HER2), erbB-3 (HER3) and erbB-4 (HER4) (Ullrich and Schelessinger, 1990). Of particular relevance to this annual report is the observation that elevated expression of the EGFR family members has been frequently observed in a significant proportion of human breast cancers. For example, amplification and consequent overexpression of Neu has been observed in a breast cancers of the comedo type (Cardiff and Muller, 1993). Indeed there is also evidence to suggest that overexpression of Neu in human breast cancers is inversely correlated with the survival of the patient (King et al., 1985, Slamon et al., 1987, Slamon et al., 1989, Gullick et al., 1991, Paterson et al., 1991). In addition elevated expression of EGFR, erbB-3 and erbB-4 have been implicated in the genesis of human breast cancer (Kraus et al., 1989, Plowman et al., 1990, Plowman et al., 1993).

In addition to the detection of elevated expression of these RTKs, many of the ligands for the EGFR family members are expressed in both primary breast cancers and their derived cell lines. For example, expression of EGFR ligands such as EGF and TGFα can also result in the activation of the other EGFR family through the formation of different heterodimers comprising EGFR and the other members of EGFR family (Stern and Kamps, 1988, Goldman et al., 1990, Kokai et al., 1988, Kokai et al., 1989, Wada et al., 1990). Although these other EGFR family members cannot bind these EGF ligands, they are transphosphorylated by the activated EGFR following ligand stimulation. Indeed coexpression of EGFR with Neu RTKs results in efficient transformation of a variety of cell lines (Kokai et al., 1989)

Direct evidence for the involvement of the EGFR family in the induction of mammary carcinoma derives from observations with transgenic mice that have been engineered to overexpress the Neu RTK (Muller et al., 1988, Bouchard et al., 1990, Guy et al., 1992). Initial studies with transgenic mice expressing a constitutively active form of neu under the transcriptional control of the mouse mammary tumour virus promoter/enhancer suggested that activation of Neu was sufficient for the single step induction of mammary tumours that affected every female transgenic carrier analyzed (Muller et al., 1988). Consistent with these observations, retroviral transfer of activated neu in the mammary epithelium of rats also led to rapid development of mammary tumours (Wang et al., 1990). By contrast to these observation expression of the neu proto-oncogene in the mammary epithelium of transgenic mice results in the stochastic appearance of focal mammary tumours that frequently metastasize to the lung. Biochemical analyses of these mammary tumours revealed that the acquisition of the transformed phenotype

was correlated with increase in the intrinsic tyrosine kinase activity of neu, and the appearance of several tyrosine phosphorylated proteins (Guy et al. 1992). In large percentage of these mammary tumours, the increase in the catalytic activity of Neu occurs as a result of activating mutations located in the transgene (Siegel et al., 1994). Taken together these observations suggest that activation of the intrinsic tyrosine kinase activity of Neu is a pivotal step in the initiation of mammary tumorigenesis.

Evidence supporting the direct role for the other EGFR family members in mammary tumorigenesis derives from observations with transgenic mice expressing EGFR ligand, TGFα in the mammary epithelium. In several independent strains of transgenic mice mammary epithelial expression of TGFα resulted in the induction mammary epithelial hyperplasias (Matsui et al., 1990, Sandgren et al., 1990, Jhappan et al., 1990) that eventually progress further into focal mammary adenocarcinomas. These observations suggest that activation of EGFR can result in deregulated mammary epithelial proliferation. Several recent studies have suggested that activation of the EGFR is also required normal mammary epithelial development. In those studies, analyses of a naturally occurring mouse mutant known as Waved-2 (Lutteke et al., 1993) which carries a kinase defective EGFR exhibits a profound lactation defect (Fowler et al., 1995). Thus activation of the EGFR family play an important role in normal mammary epithelial proliferation.

The purpose of present research is to investigate the role of the various EGFR family in the induction of mammary carcinoma. Our first research objective was to assess whether coactivation of the EGFR and Neu in the mammary epithelium results in the acceleration in the induction of mammary tumours. Because the EGFR and Neu are capable of forming heterodimers that are responsive to EGF ligands such as TGFα, we examined whether coexpression of TGFα and Neu could act synergistically to transform the mammary epithelium. This was accomplished by crossing the separate transgenic strains carrying the MMTV/TGFα and MMTV/neu fusion gene to derive dual transgene carriers that coexpress TGFα and Neu in the mammary epithelium. The results of these analyses revealed that by contrast to the parental strains which developed focal mammary tumours with long latency, the dual carriers developed multifocal mammary tumours with accelerated kinetics. As expected, the rapid induction of mammary tumours in the dual carriers correlated with the appearance of tyrosine phosphorylated Neu and EGFR. These data suggest that coactivation of Neu and EGFR can dramatically accelerate the induction of mammary carcinoma in these transgenic strains via a mechanism involving receptor transactivation.

The second research objective of the initial funding year was to elucidate a potential mechanism to explain the observed cooperativity between EGFR and Neu. One potential means by which this could occur is that the EGFR and Neu recruit distinct but complementary signalling pathways that can cooperate to transform the mammary epithelial cell. To this end we have recently demonstrated that Neu but not the EGFR can directly interact and activate the c-Src tyrosine kinase in a tyrosine phosphorylation dependent manner. Moreover EGF stimulation of the EGFR can activate c-Src indirectly through transphosphorylation of Neu (Muthuswamy and Muller, 1995). These data suggest that TGFα and Neu may cooperate through the recruitment of the c-Src signalling pathway.

In addition to these studies, we are in the process of determining whether a functional EGFR was required for the induction of mammary tumours by Neu. To test this hypothesis we are currently interbreeding the Waved-2 mouse mutant with transgenic mice expressing either the wild-type or constitutive activated version of Neu.

RESULTS

Synergistic Interaction of the Neu proto-oncogene and TGFα in the mammary epithelium of transgenic mice.

To explore whether TGFα and Neu could cooperate in mammary tumorigenesis transgenic mice bearing the MMTV/TGFα and MMTV/wild-type neu transgenes were interbred to generate F1 mice that carried either neu, TGFα or both transgenes. These studies were done in close collaboration with the laboratory of Dr. Robert Coffey at Vanderbilt university. Because the TGFα females were unable to nurse their young, these F1 progeny were generated by crossing MMTV/TGFα males with MMTV/neu females. The MMTV/TGFα mice were derived from line 29 strain (Matsui et al., 1990) whereas the MMTV/neu mice are derived from the N#202 lineage (Guy et al., 1992). The genotypes of the various progeny were confirmed by Southern blot analyses on genomic tail DNA with probes specific to the TGFα or the neu transgenes (Matsui et al., 1990, Guy et al., 1992).

To determine if coexpression of TGFα and Neu could accelerate the occurrence of mammary tumours in bigenic animals virgin female mice were monitored for the development of mammary tumours by physical palpation. As shown in Figure 1 (Appendix #1), mammary tumours in either the MMTV/neu or MMTV/TGFα strains occurred only after a long latency period and were focal in origin. For example, 6% of the MMTV/TGFα and 35% of the MMTV/neu mice had developed

mammary tumours by 250 days of age (Figure 1, Appendix #1). In marked contrast 95% of the bitransgenic virgin mice developed mammary tumours by this point. Indeed, 50% of the dual carriers had developed mammary tumours at 175 days of age whereas neither single transgene carrier had yet developed mammary tumours. (Figure 1, Appendix #1). In addition to the accelerated onset of mammary tumours, the tumours that arose in the dual transgene carriers were generally multifocal in origin.

To further investigate the morphological differences between single and dual transgene carriers, the mammary epithelium from age-matched virgin carriers were subjected to wholemount analyses (Vonderharr and Greco, 1979). As shown in Figure 2 (Appendix 1), the results showed that virgin mice carrying the neu transgene, morphologically resembled the mammary ductal structure from virgin FVB/N mice (Figure 2B, Appendix #1). By contrast, the virgin mammary ductal structures from either the TGFα or Neu/TGFα were clearly abnormal (compare Figure 2C and 2D). Comparison of these virgin ductal structures to mammary epithelium of a normal lactating FVB/N mice (Figure 2A) revealed that like the lactating mammary gland these wholemounts displayed extensive lobular-alveolar development. However, close inspection of the TGFα and Neu/TGFα whole mounts showed that they also possessed distinctive differences. For example, the alveoli of the Neu/TGFα whole mount possessed a denser cell lining compared to the cystically dilated alveoli found in the TGFα virgin mice (compare Figures 2C and 2D). Consistent with this whole mount analyses, histological examination of the mammary epithelial hyperplasias from both TGFα and Neu/TGFα mice showed that only in the latter could epithelial dysplasias also be detected (compare Figures 3C and 3D, appendix 1). Interestingly, both TGFα and Neu/TGFα mammary glands displayed evidence of inflammatory stromal tissue that was absent in the Neu or FVB/N mammary glands (Figure 3, Appendix 1). These observations argue that the appearance of the inflammatory stroma correlates with the detection of the TGFα transgene. Taken together, these observations suggest that by comparison to single transgene bearing animals, those possessing both transgenes develop widespread histological abnormalities of the mammary gland.

The induction of mammary epithelial hyperplasias and tumours correlates with the coexpression of neu and TGFα transcripts.

To confirm that the phenotypes observed in dual transgene carriers was a result of coexpression of neu and TGFα transgene products, RNase protection analyses (Melton et al., 1984) with probes specific for TGFα, Neu, and EGFR (see Matsui et al., 1990,

Siegel et al., 1994) were conducted on 10ug of total RNA derived from the mammary tissue samples of the various transgene carriers. In addition to ensure that equal quantities of RNA were analyzed, a phosphoglycerate kinase antisense probe (Mori et al., 1986) was also included in the analyses. RNA was isolated using the protocol described by Chirgwin et al., 1979. The results of these analyses are summarized in Figure 4 (Appendix #1). As shown in Figure 4A, hybridization of the mammary tissue RNA samples with an antisense neu riboprobe revealed abundant neu transcripts from animal carrying the neu alone or both neu and TGFα transgenes (Figure 4A). Interestingly in tumours induced by the Neu alone (Figure 4A, lanes 2, 4), altered transcripts corresponding to in frame deletions in the juxtatransmembrane domain were detected a reported previously (Siegel et al., 1994). Significantly, these altered transcripts were not detected in tumours derived from the biogenic animals (Figure 4A; lanes 9-11). By contrast to these observations neu transcripts were not detected in hyperplastic or tumour tissues derived from the MMTV/TGFα mice (Figure 4A). As expected TGFα transcripts were detected in tumour or hyperplastic tissues derived from the TGFα or Neu/TGFα tissues and were beyond detection limits in the Neu-induced mammary tumours (Figure 4B, Appendix #1). In concert with the detection of TGFα transcripts comparable levels of EGFR were also detected in these tissues (Figure 4C; lanes 5-11).

To explore whether coexpression of TGFα and Neu in the mammary epithelium of transgenic mice resulted in the concerted activation of the Neu and EGFR RTKs protein lysates from these same tissues were immunoprecipitated with either Neu or EGFR specific antisera and immunoblotted with antiphosphotyrosine antibodies (Figure 5, Appendix #1). As shown in Figure 5A, immunoprecipitation protein lysates with Neu specific antibodies followed by immunoblot analyses with antiphosphotyrosine antibodies demonstrated the presence of tyrosine phosphorylated Neu in mammary tumours derived from tumours derived from animals expressing the neu transgene alone or from animals expressing both transgenes (Figure 5A, lanes 1,3,7,8). As expected from the RNase protection analyses (Figure 4, Appendix #1), we did not detect tyrosine phosphorylated Neu in tumour samples expressing TGFα alone (Figure 5A, lanes 5 and 6). Analyses of the same set of samples for tyrosine phosphorylated EGFR revealed that the tumour samples expressing TGFα alone (Figure 5B, lanes 5-6) and coexpressing TGFα and Neu (Figure 5B, lanes 7-8) possessed significant levels of tyrosine phosphorylated EGFR. (Figure 5C, lanes 5-8). These data argue that the dramatic synergism observed between TGFα and Neu in tumour induction correlates with the coactivation of EGFR and Neu.

Activation of c-Src by the activated EGFR and Neu RTKs correlates with the direct and specific interaction of c-Src and Neu.

Although the interbreeding of the TGFα and Neu transgenic mice strongly suggest that the activated EGFR and Neu can cooperate in the induction of mammary tumours, the molecular basis for this cooperativity is unclear. One signalling pathway that appears to play a key role in the induction of mammary tumours is that involving the c-Src tyrosine kinase. For example, EGF stimulation of cells expressing the EGFR results in a modest elevation of Src family members including c-Src (Osherov and Levitzki, 1994, Muthuswamy and Muller, 1995). Moreover, coexpression of EGFR and c-Src results in hyperresponsive proliferative response both *in vivo* and *in vitro* (Wilson et al., 1989, Ma et al., 1995). In fact we and others have demonstrated that c-Src activity is elevated in mammary tumor cells expressing activated Neu and have further demonstrated that this activation correlates with the formation of c-Src and Neu complexes *in vivo* (Muthuswamy et al., 1994, Luttrel et al., 1994, Muthuswamy and Muller, 1995). Further evidence supporting this assertion derives from observation that mice c-Src activity is required for the induction of mammary tumors by mice express PyV middle T antigen (Guy et al., 1994).

To test the capacity of a radiolabeled c-Src fusion protein to bind the activated Neu and EGFR, we immunoprecipitated the activated EGFR following EGF stimulation from two EGFR overexpressing lines and Neu from a mammary tumor cell line expressing an activated form of Neu (Muthuswamy et al., 1994). As shown in Figure 6A (Appendix #1), direct binding of c-Src could be detected in cells expressing activated Neu but did not interact with the EGFR immunoprecipitates. The inability to detect binding of c-Src to the activated EGFR was not due to a lack of tyrosine phosphorylated EGFR since comparable levels of tyrosine phosphorylated EGFR and Neu RTKs could be detected (Figure 6C, Appendix #1). These observations suggest that direct interaction of c-Src occurs with activated Neu but cannot be detected with the activated EGFR.

One possible explanation for these observations is that activation of c-Src by the activated EGFR occurs through the transactivation of the Neu by the activated EGFR as previously reported by others (Stern and Kamps, 1988, Kokai et al., 1989, Wada et al., 1990). To test this hypothesis, we examined whether physical complexes between c-Src and Neu could be detected in cells overexpressing EGFR following EGF stimulation. To accomplish this goal, we initially immunoprecipitated protein lysates from these cells with antibodies directed against EGFR, Neu and c-Src and the immunoblotted these immunoprecipitates with antiphosphotyrosine specific antibodies (Figure 7A, Appendix #1). As expected treatment of the EGFR overexpressing cells with EGF resulted in the induction

of a tyrosine phosphorylated band that comigrated with the expected molecular weight of the EGFR (Figure 7A, lane 2). In addition to activating the EGFR EGF stimulation also resulted in the transphosphorylation of Neu (Figure 7A, lane 4). Interestingly, analyses of c-Src immunoprecipitates revealed that EGF stimulation resulted in the formation of a complex between c-Src and a 185 kDa tyrosine phosphorylated protein that comigrated with Neu but not the EGFR (Figure 7, lane 6. To confirm that 185 kDa tyrosine phosphorylated protein was in fact Neu, the same immunoprecipitates were immunoblotted with either Neu specific antisera (Figure 7B) or EGFR specific antibodies (Figure 7C). These experiments confirmed that 185 kDa c-Src associated protein was in fact Neu (Figure 7B ,lane 6). Taken together these observation suggest that EGF mediated activation of c-Src occurs through a direct and specific interaction of c-Src with Neu.

CONCLUSIONS AND FUTURE DIRECTIONS

The results presented show that coexpression of Neu and TGFα in the mammary epithelium of transgenic mice results in the rapid induction of multifocal mammary tumors. We also present data which suggests that underlying synergism observed between the TGFα and Neu may involve the ability of Neu to directly recruit the c-Src tyrosine kinase upon its transactivation by the activated EGFR.

The rapid induction of mammary tumour observed in animals carrying both TGFα and neu transgenes correlates with the elevated levels of both transgene transcripts. By contrast to Neu-induced mammary tumor transcripts where altered transcripts are frequently detected(Figure 4A, lanes 2 and 4, Siegel et al., 1994), tumours coexpressing both TGFα and Neu fail to demonstrate any evidence of altered transcripts. (Figure 4A, lanes 9-11). However, significant levels of tyrosine phosphorylated Neu were detected in mammary tumours expressing Neu or both Neu and TGFα (Figure 5A, lanes 1 and 3, lanes 7-8, Appendix #1). These observations suggest that activation of Neu in the Neu/TGFα tumors occurs in through a different mechanism than that in tumors induced by Neu alone. A likely explanation for this difference is that activation of Neu in the tumours coexpressing both TGFα and Neu occurs through its transactivation by the activated EGFR. Indeed, unlike mammary tumours induced by Neu alone, significant levels of tyrosine phosphorylated EGFR can be detected in tumors coexpressing TGFα and Neu (Figure 5C , lanes 8-9). Consistent with this hypothesis, we and others have demonstrated that Neu can be transactivated by the EGFR following EGF stimulation (Akiyam et al., 1988, Stern and Kamps, 1988, Kokai et al., 1988, Wada et al., 1990, Goldman et al.,

1990). Because EGFR transcripts and protein are beyond the range of detection in Neu induced mammary tumors there is likely a stronger biological selection for the occurrence of activating mutations in the transgene.

Interestingly, the levels of tyrosine phosphorylated Neu in the Neu/TGFα coexpressing tumours is much lower than that observed in Neu tumors alone (Figure 5). One possible explanation for these data is that lower levels of tyrosine phosphorylated are required due to the concerted activation of the EGFR. In fact, it has been demonstrated that EGFR and Neu can cooperate to transform Rat-1 fibroblasts (Kokai et al., 1990). The observed cooperativity of Neu and EGFR may be due the ability of these closely related RTKs to recruit distinct but complementary signalling pathways. Consistent with this hypothesis, several studies have suggested that coupling of the phosphahaditylinositol-3' kinase with the EGFR requires the participation of c-erbB-3 (Sotloff et al., 1994, Pringent and Gullick, 1994). Moreover we have demonstrated that activation of c-Src by EGFR at least in fibroblastrs requires the function of the activated Neu RTK (Figures 6 and 7, Appendix #1). Whether the potent transforming activity exhibited by coexpression of TGFα and Neu in the mammary epithelium results from recruitment of the c-Src signalling pathway awaits further analyses.

One important question that remains to be addressed in this system is whether the observed cooperativity between TGFα and Neu is due to the formation of Neu/EGFR heterodimers. However, we as well as our collaborators (Dr. Robert Coffey and Dr. Carlos Arteaga) have not yet been able to detect stable Neu/EGFR heterodimers using both immunoprecipitation/immunoblot analyses or via chemical crosslinking experiments (data not shown). These data argue that if heterodimerization between EGFR and Neu is occurring in the Neu/TGFα coexpressing tumors, the formation of these complexes is likely transient in nature. Nonetheless our observations strongly suggest that TGFα and neu cooperate in mammary tumorigenesis through a mechanism involving receptor transactivation.

Although these observations suggest that TGFα cooperates with Neu through an activated EGFR, another important question that remains to be addressed is whether activation of EGFR is necessary for Neu induced mammary tumorigenesis. To examine the role of EGFR, we are in the process of interbreeding the transgenic mice expressing either wild type neu (Guy et al., 1992) with a naturally occurring mouse mutant known as waved-2 which possesses a mutation in the EGFR catalytic domain rendering the EGFR functionally inactive (Luetteke et al., 1994). The results of these experiments should allow us to address whether EGFR function is required for the induction of mammary tumours in these mice.

In addition to our focus on Neu and TGFα, we are also interested in examining the function of the other members of the EGFR family in mammary tumorigenesis. To this end we have constructed MMTV-driven expression cassettes bearing the other members of EGFR family including the EGFR, c-erbB-3 and c-erbB-4 (Figure 8, Appendix #1 and are now in the process of deriving transgenic mice with these constructs. Once an appropriate number of founder transgenic lines have been establish, we will establish whether overexpression of these other EGFR family members is capable of inducing mammary tumour. The generation and characterization of these lines will be the major focus during the upcoming year.

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APPENDIX #1

FIGURE LEGENDS

FIGURE 1. Kinetics of tumour occurrence in monogenic and bigenic animals harboring the MMTV/TGFα and MMTV/neu transgenes. Comparison of the kinetics of tumour formation between virgin female carriers bearing the MMTV/TGFα, MMTV/neu and both transgenes. The age at which 50% of the mice were found to have tumours (T50) and the number of mice examined (n) are indicated.

FIGURE 2. Wholemount analyses of mammary fat pads derived from monogenic and bigenic female mice. Whole mount preparations at 31.5x magnification illustrating the comparative subgross appearance of mammary trees from: (A) Lactating FVB female (B) Virgin female with neu transgene. Note the numerous side buds which give the mammary tree a spiculated appearance. (C) Virgin female with the TGFα transgene Note the well developed, cystically dilated alveoli. (D) Virgin female with both TGFα and neu transgenes. Note the larger cystic alveoli with darker walls, indicating a denser cell lining in the walls. Compare these preparations with comparable histologic preparations in Figure 4.

FIGURE 3. Histopathology of mammary tissue derived from virgin monogenic and bigenic transgenic animals: (A) Normal FVB lactating female mouse showing lobuloalveolar development and milk production. (B) Transgenic neu virgin female mouse illustrating rudimentary mammary acinar development without significant luminal secretions. (C) Transgenic TGFα virgin female mouse illustrating extensive alveolar development in comparison with lactating mammary gland (A) Note that the alveoli are much more distended with secretory products than the FVB lactating tissue but contain fewer clear lipid vacuoles. (D) Transgenic TGFα/neu virgin female mouse illustrating areas of alveolar development with papillary hyperplasia in the upper right corner. The virgin neu, TGFα and Neu/TGFα were age matched (139 days) and identical to those described in Figure 3.

FIGURE 4. Expression of neu and TGFα transgenes in mammary tissue of transgenic mice: (A) Neu transgene expression in mammary tissues of mice carrying the MMTV/neu transgene (neu/+), TGFα transgene (TGFα/+) and both transgenes (neu/TGFα). Tissue RNA samples derived from tumour tissue (BT) and adjacent mammary tissue (NB) were subjected to RNase protection analyses. The protected wild type (WT) neu transcript is 640 nucleotides in length. Protected

fragments corresponding to the altered neu transcript are indicated by the arrows. Tissue RNA samples from #5861 and #5862 were derived from virgin female animals which exhibited extensive mammary epithelial hyperplasias. Tissue RNA samples from #5368 and # 5359 were derived from mammary epithelial hyperplasias from multiparous female animals whereas RNA samples derived from #5367 and #4545 were derived from tumour bearing multiparous female carriers. An antisense riboprobe, directed against the mouse phosphoglycerate kinase gene, was used to control for equal loading of RNA on the gel. The PGK-1 probe protects a 124-nucleotide fragment as indicated in the lower panels. (B) The identical RNA tissue samples were probed with a antisense probe directed against the mouse TGFα gene. The TGFα antisense prober protects a 632 nucleotide fragment. (C) The identical RNA tissue samples were probed with an antisense probe directed against the murine EGFR.

FIGURE 5. Mammary tissue from the bigenic Neu/TGFα mice possess constitutively activated Neu and EGFR RTKs: (A) Protein lysates from tumour tissue (BT) and adjacent mammary epithelial tissue (NB) carrying either the MMTV/neu transgene(neu/+), the MMTV/TGFα transgene (TGFα/+) or both transgenes (neu/TGFα) were immunoprecipitated (IP) with a monoclonal antibody (7.16.4) (anti-Neu) and then subjected to an immunoblot analysis (Blot) with an antiphosphotyrosine antibody (4G10) (anti-ptyr). The position of the tyrosine phosphorylated Neu protein is indicated by the arrow. (B) The identical tissue lysates were subjected to immunoblot analyses (Blot) with anti TGFα monoclclonal (see Material and Methods). (C) The identical protein lysates were immunoprecipitated (IP) with anti-EGFR antibody (see Material and Methods) and then subjected to an immunoblot analysis with an antiphosphotyrosine antibody (4G10) (anti-ptyr). The position of the tyrosine phosphorylated EGFR protein is indicated by the arrow. The tissues were derived from the same samples described in Figure 1.

FIGURE 6. The c-Src SH2 domain does not interact directly with tyrosine phosphorylated EGFR. Lysates from EGF treated A431 epithelial cells (lane 2) or R1/hER fibroblasts (lane 4) were immunoprecipitated with anti-EGFR antibodies (anti-EGFR) and Neu was immunoprecipitated from NAFA cell lysates (anti-Neu). (A) One half of the immunoprecipitates was resolved on a SDS-PAGE and probed with radiolabeled c-Src SH2 domain (GSTag-c-Src SH2). (B) The remainder of the immunoprecipitate was immunoblotted with anti-phosphotyrosine antibody (anti-pTyr). Normal mouse serum (NMS, lane 1) was used as a nonspecific control. (IP: Immunoprecipitation). The molecular weight markers are in kDa.

FIGURE 7. EGF treatment results in specific association of c-Src with Neu. Lysates were derived from R1/hER both before (-) and after (+) one minute induction with EGF. EGFR (anti-EGFR) or Neu (anti-Neu) or c-Src (Anti-cSrc) were immunoprecipitated from lysates and resolved on a SDS-PAGE gel. The immunoprecipitates were probed with anti-phosphotyrosine (A) or anti-Neu (B) or with anti-EGFR (C) antibodies. The autoradiograph in panel C was exposed almost 10 times longer than that in B.

FIGURE 8. A schematic illustrating the structure of transgenes for each of the EGFR family members. The unshaded region represents sequences with the pBluescript KS vector backbone, the striped region contains the Mouse Mammary Tumour Virus-Long Terminal Repeat (MMTV-LTR) derived from the plasmid pA9, while the stippled region immediately following the MMTV-LTR corresponds to an inert region derived from the original pA9 vector. In each of the illustrated constructs, the black region indicates the position of the various erbB family members whereas the adjacent sequence depicted in gray contains the SV40 polyadenylation cassette. The restriction sites used to release the injection fragment are indicated below each construct.

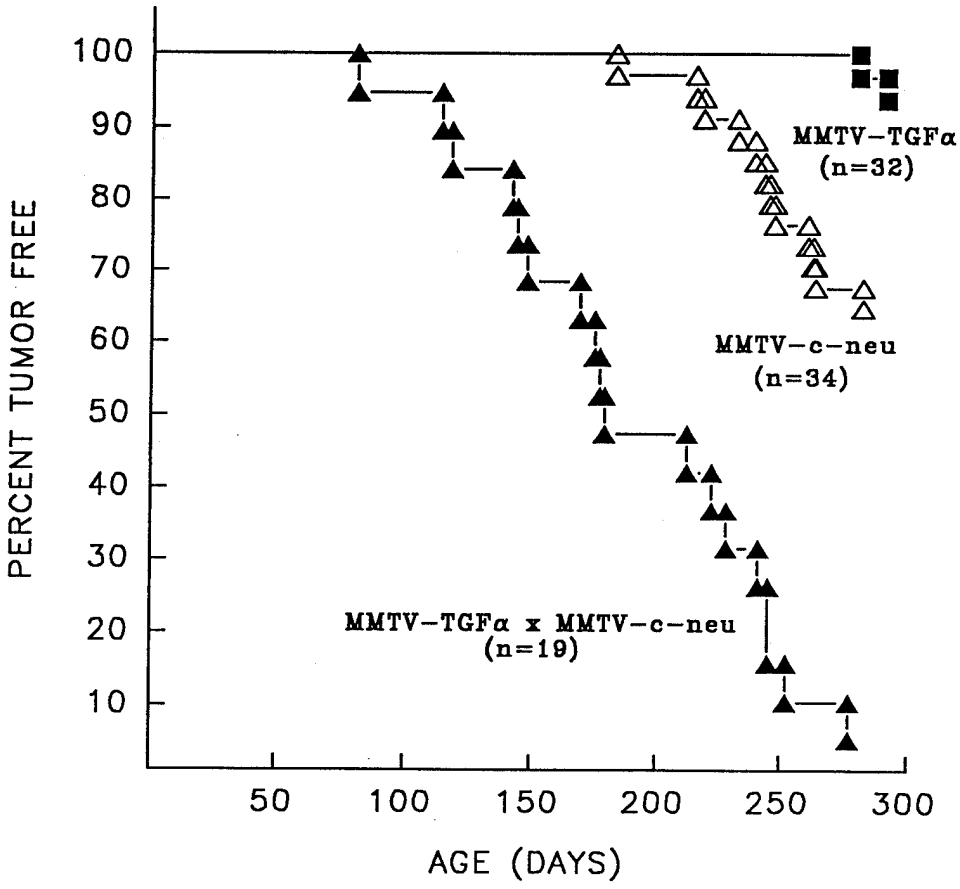
100
90
80
70
60
50
40
30
20
10
PERCENT TUMOR FREE
50
100
150
200
250
300
AGE (DAYS)
MMTV–TGFα
(n=32)
MMTV–c–neu
(n=34)
MMTV–TGFα x MMTV–c–neu
(n=19)

FIGURE 1

MULLER, William J.

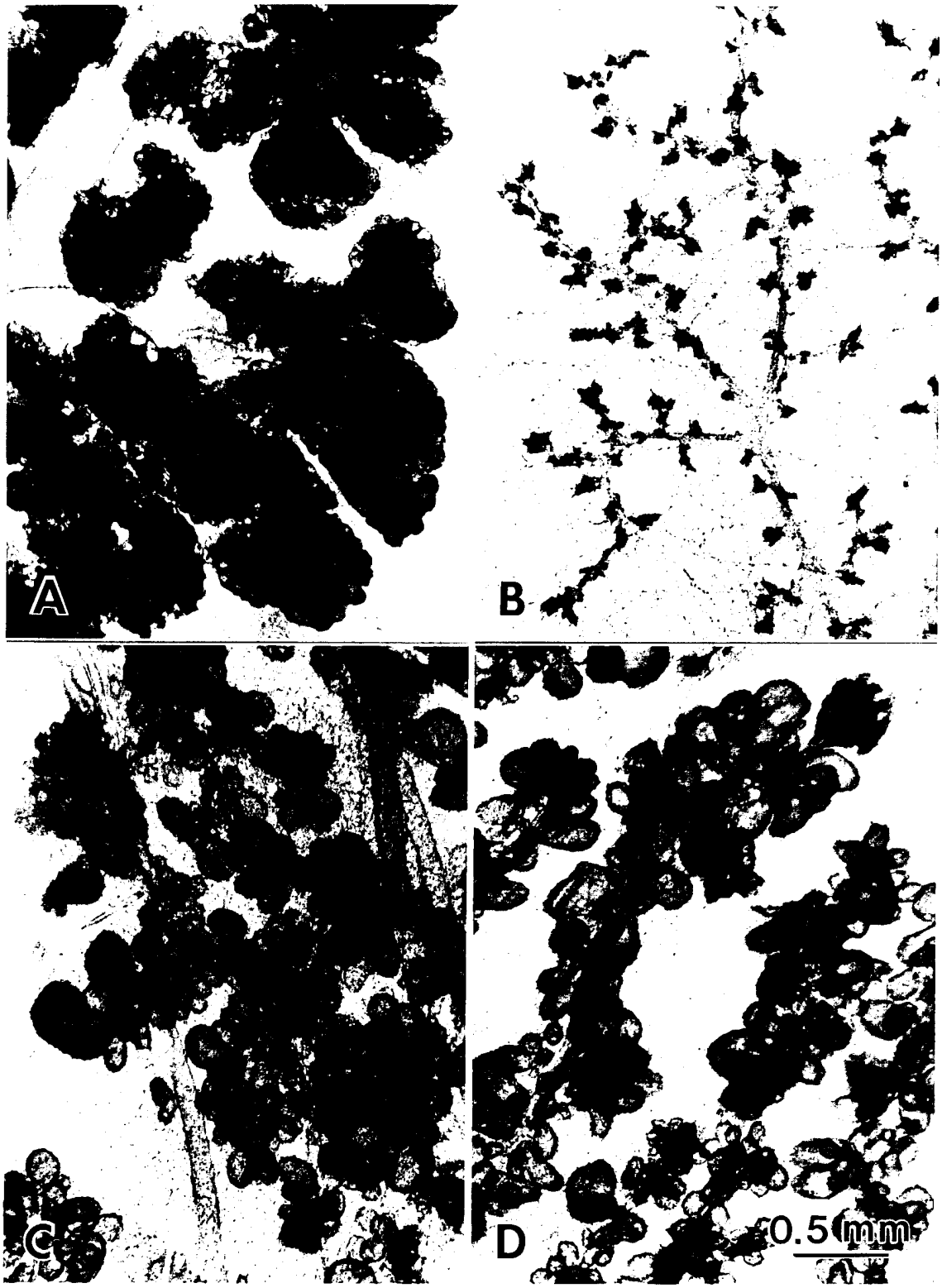
A
B
C
D
0.5 mm

FIGURE 2

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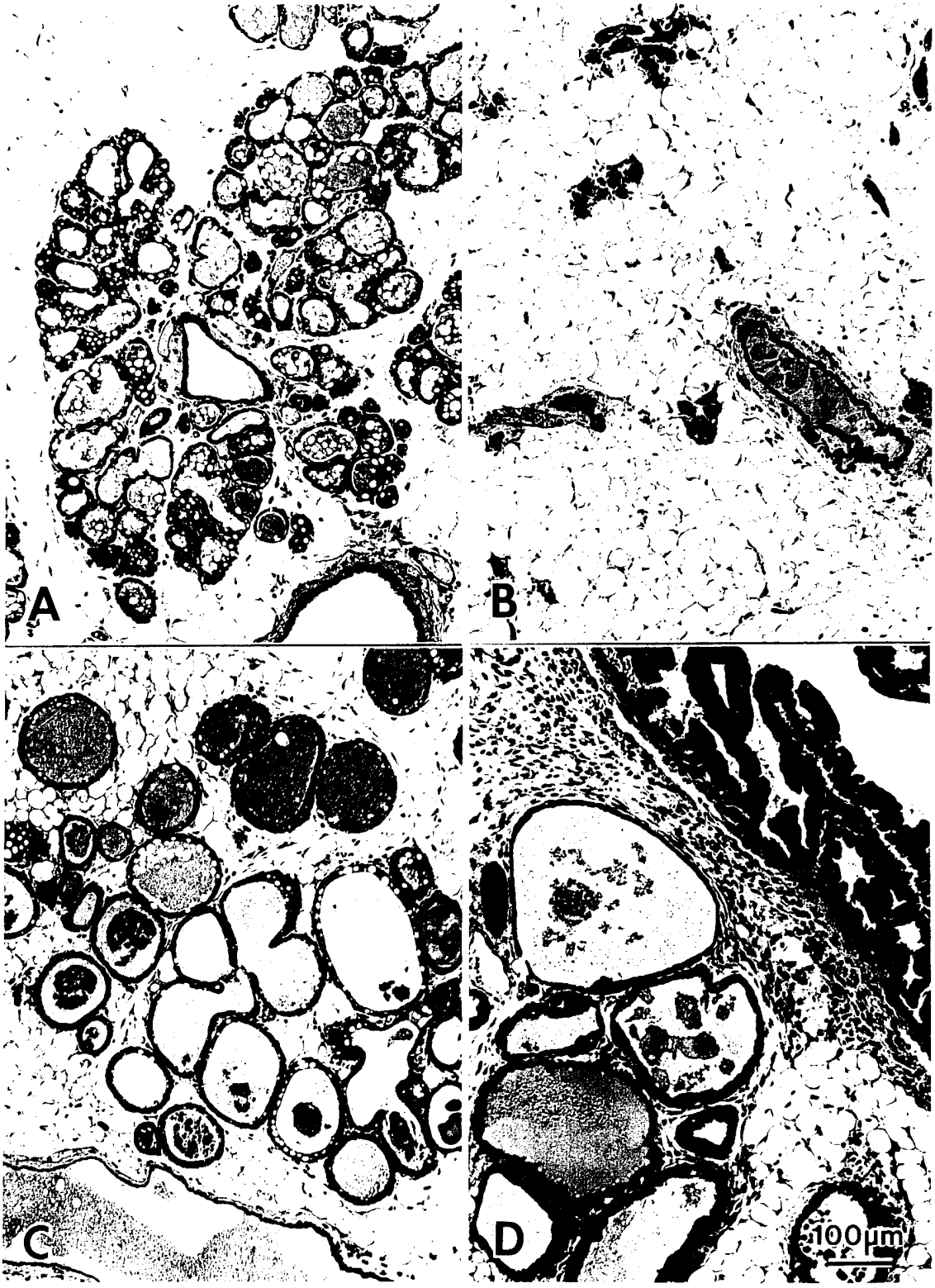
A
B
C
D
100µm

FIGURE 3

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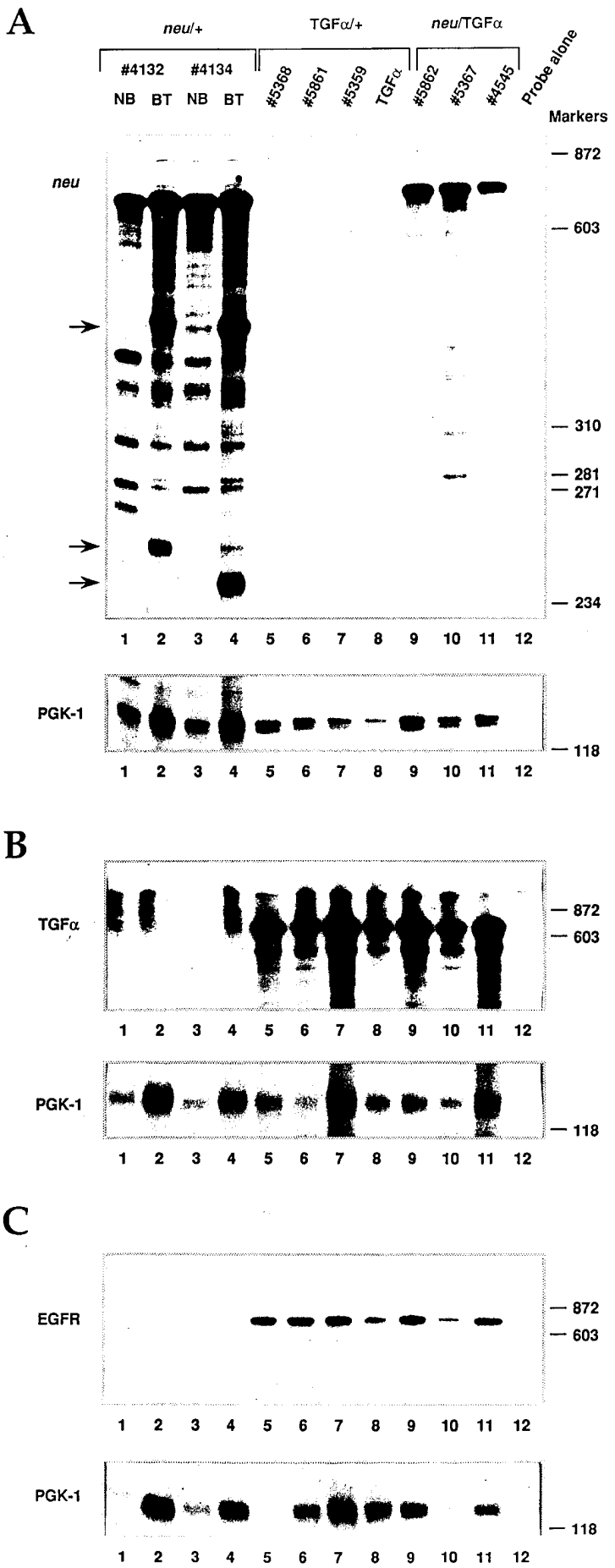
A
neu/+
TGFα/+
neu/TGFα
#4132
#4134
NB
BT
NB
BT
#5368
#5861
#5359
TGFα
#5862
#5367
#4545
Probe alone
Markers
neu
872
603
310
281
271
234
1 2 3 4 5 6 7 8 9 10 11 12
PGK-1
118
1 2 3 4 5 6 7 8 9 10 11 12
B
TGFα
872
603
1 2 3 4 5 6 7 8 9 10 11 12
PGK-1
118
1 2 3 4 5 6 7 8 9 10 11 12
C
EGFR
872
603
1 2 3 4 5 6 7 8 9 10 11 12
PGK-1
118
1 2 3 4 5 6 7 8 9 10 11 12

FIGURE 4

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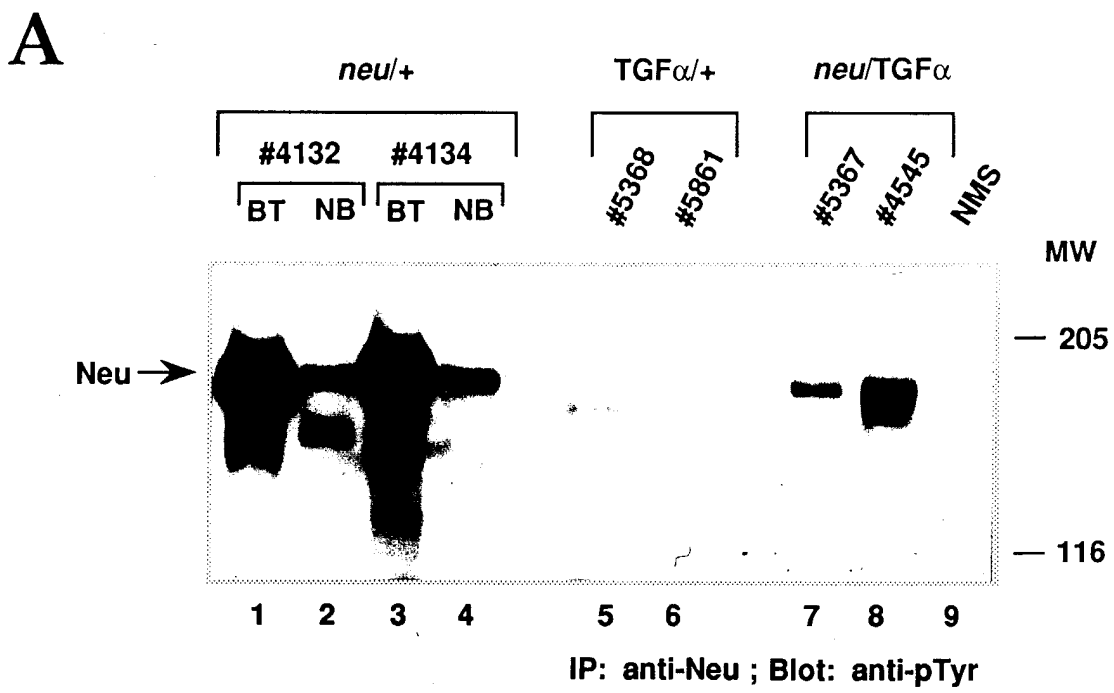
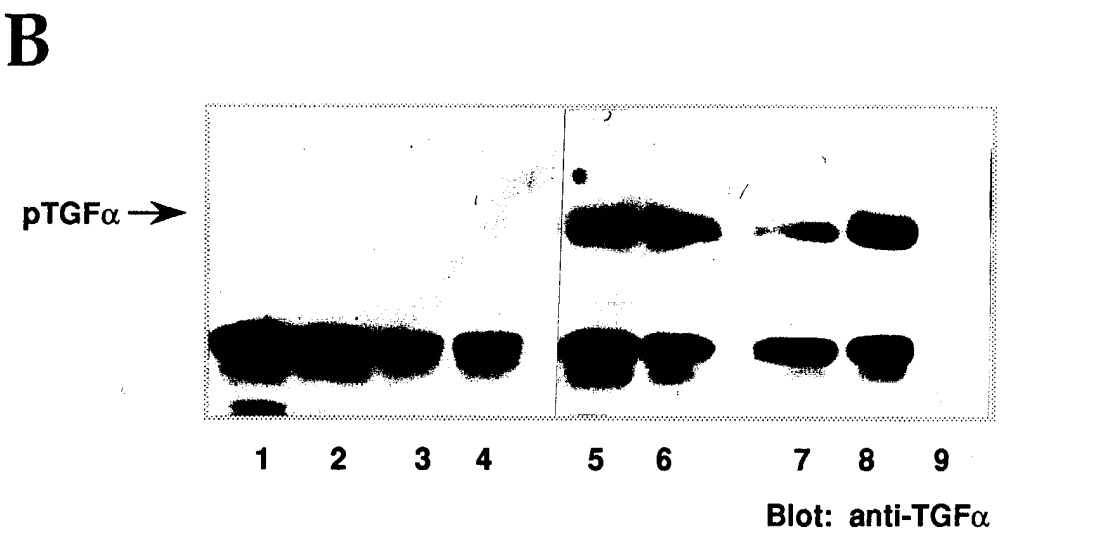

A
neu/+
TGFα/+
neu/TGFα
#4132
#4134
BT
NB
BT
NB
#5368
#5861
#5367
#4545
NMS
MW
Neu
205
116
1 2 3 4 5 6 7 8 9
IP: anti-Neu ; Blot: anti-pTyr


B
pTGFα
1 2 3 4 5 6 7 8 9
Blot: anti-TGFα

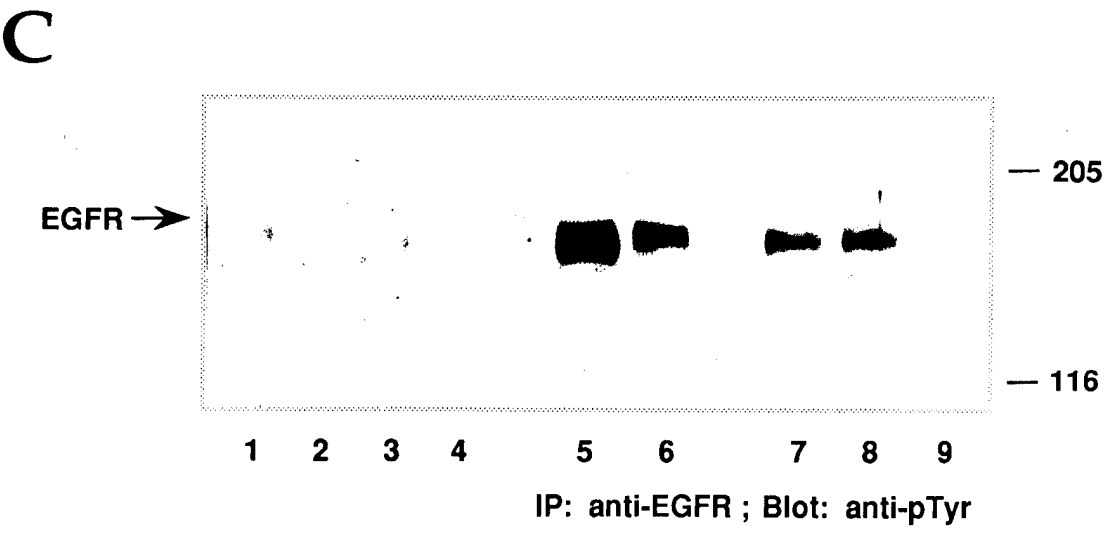

C
EGFR
205
116
1 2 3 4 5 6 7 8 9
IP: anti-EGFR ; Blot: anti-pTyr

FIGURE 5

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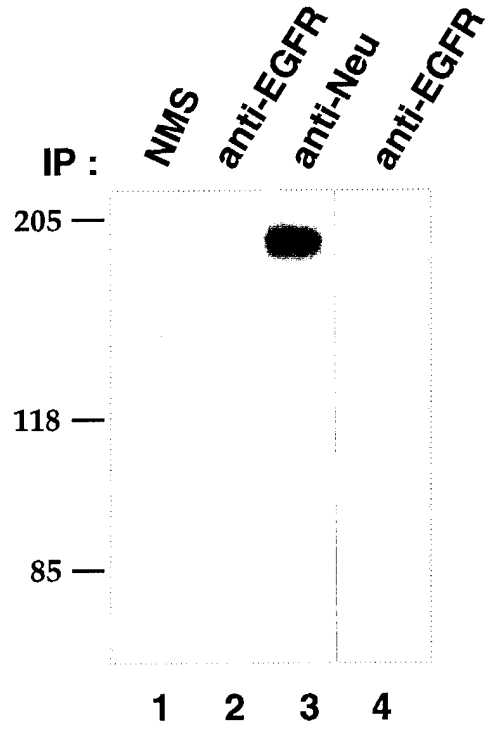
A



Probe: GSTag-c-Src SH2

B

Blot: anti-pTyr

FIGURE 6

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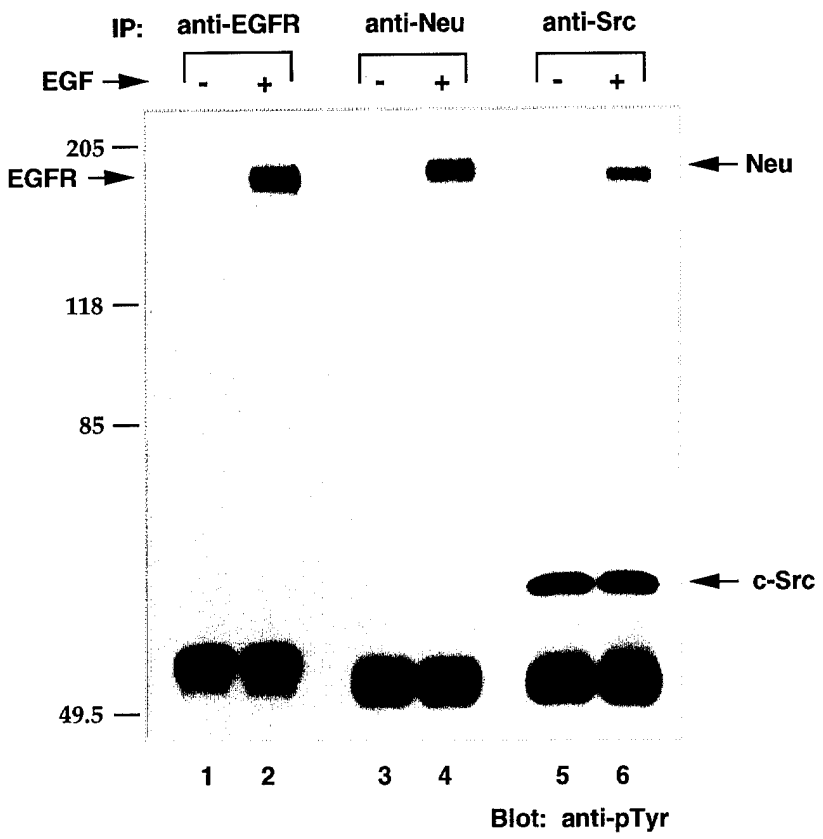
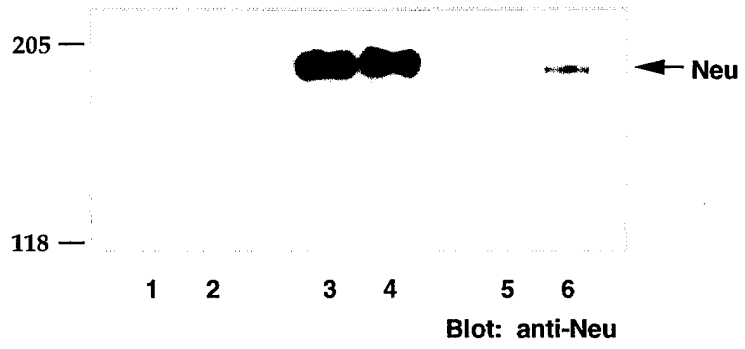
A


IP:
anti-EGFR
anti-Neu
anti-Src
EGF
-
+
-
+
-
+
205
EGFR
118
85
49.5
Neu
c-Src
1
2
3
4
5
6
Blot: anti-pTyr

B


205
118
Neu
1
2
3
4
5
6
Blot: anti-Neu

C

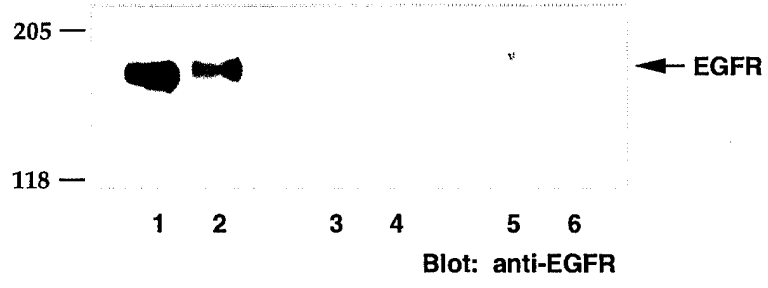

205
118
EGFR
1
2
3
4
5
6
Blot: anti-EGFR

FIGURE 7

MULLER, William J.

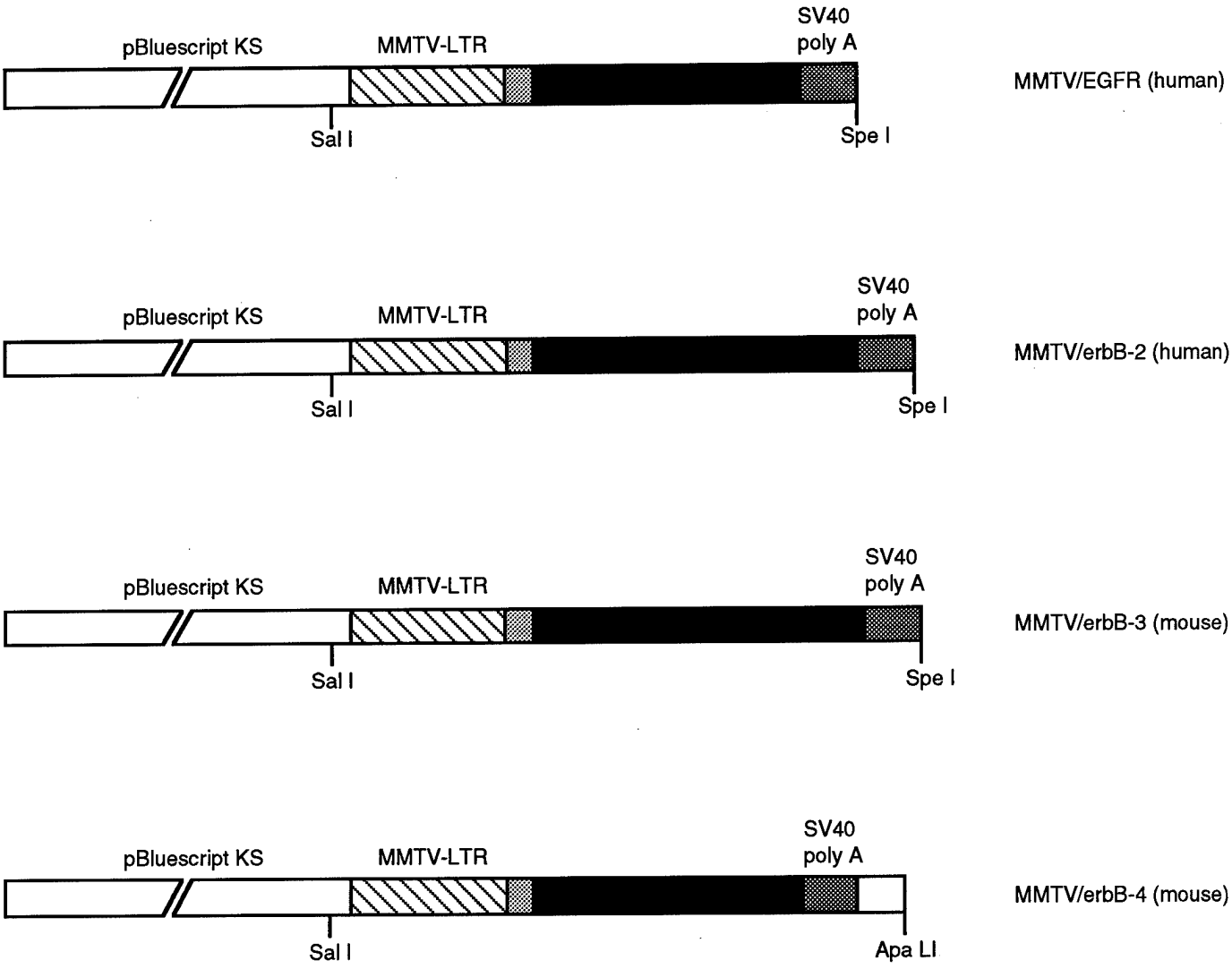

pBluescript KS
MMTV-LTR
SV40
poly A
Sal I
Spe I
MMTV/EGFR (human)
pBluescript KS
MMTV-LTR
SV40
poly A
Sal I
Spe I
MMTV/erbB-2 (human)
pBluescript KS
MMTV-LTR
SV40
poly A
Sal I
Spe I
MMTV/erbB-3 (mouse)
pBluescript KS
MMTV-LTR
SV40
poly A
Sal I
Apa LI
MMTV/erbB-4 (mouse)

FIGURE 8

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